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Acidic fibroblast growth factor enhances regeneration of processes by postnatal mammalian retinal ganglion cells in culture

(neurotrophic factor/central nervous system/neurite extension)

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ABSTRACT **Postnatal rat retinal ganglion cells (RGCs) were identified with specific fluorescent labels and placed in culture. Under these conditions, the outgrowth of processes by RGCs was found to be promoted to a far greater degree by acidic fibroblast growth factor (aFGF) than by basic fibroblast growth factor (bFGF). The effect of aFGF and bFGF on process extension by solitary RGCs was quantified after 24 hr in culture, a time when neither aFGF nor bFGF enhanced RGC survival. The action of aFGF on process outgrowth was markedly potentiated by the addition of heparin (10 μg/ml) to the medium, but heparin alone had no effect. In the presence of heparin, half-maximal process outgrowth occurred at an aFGF concentration of <20 pg/ml (1 pM). Since all of the centrally projecting processes have already been formed in the living animal prior to use (at 7–12 days of age), at least a portion of the process outgrowth in culture appears to represent a regenerative phenomenon. Statistical analysis of the increase in process growth revealed that aFGF with heparin contributed to both neurite initiation and elongation. The mean number of glial cells, identified with polyclonal antiserum against glial fibrillary acidic protein, was slightly increased in cultures receiving aFGF plus heparin, but this effect was variable, and these glial cells were not in contact with the solitary RGCs that were scored for regeneration of processes. Thus, glial cells probably did not exert a direct physical influence on the degree of process outgrowth observed in the solitary RGCs, although a humoral effect cannot be totally excluded. These results suggest that aFGF has a potent influence on the outgrowth of processes by a neuron in the mammalian central nervous system. The potentiation of this effect by heparin leads us to speculate that the interaction of aFGF with a heparin-like molecule located in the extracellular matrix (such as heparan sulfate proteoglycan) may produce physiological effects *in vivo*. Furthermore, the lack of a substantial effect of bFGF in this system under these conditions shows that a specific population of mammalian central neurons may be differentially influenced by these two closely related peptide growth factors.**

A variety of growth factors appear to be important for the extension of processes or neurites in the mammalian nervous system. Nerve growth factor (1) is the prototype of these neurotrophic substances, but others have been characterized (2–4). Among the substances with possible neurotrophic activity in the central nervous system (CNS) are acidic and basic fibroblast growth factors, which represent distinct but homologous gene products (5, 6). Both have been shown to be potent mitogens for a variety of cell types, including endothelial cells, astrocytes, and fibroblasts (7–10). Many of the effects of acidic fibroblast growth factor (aFGF) are potentiated by heparin-like molecules to a far greater degree than the actions of basic fibroblast growth factor (bFGF) (11, 12). It has been shown that in PC12 cells aFGF and bFGF induce neurite outgrowth (11, 13). In addition, purified bFGF substantially promotes both cell survival and process extension in primary cultures of fetal rat hippocampal and cortical neurons (12, 14). In contrast, highly purified aFGF increases survival of hippocampal neurons somewhat less effectively than bFGF and exhibits only very minimal effects on process outgrowth (15).

aFGF has been isolated from bovine hypothalamus, retina, and other CNS areas; a small amount has also been found in bone (16). In contrast, bFGF has been encountered in virtually every tissue examined to date (17). Since both aFGF and bFGF are present in the adult CNS, the question naturally arises as to whether or not these potential neurotrophic factors are capable of influencing not only initial neurite outgrowth but also the regrowth of processes from injured CNS neurons. Since we originally purified aFGF from retina (10), we wanted to observe its effects on an appropriate central neuron, the retinal ganglion cell (RGC). If aFGF could stimulate the regeneration of processes by a postnatal, differentiated neuron from the mammalian CNS, it could be of clinical benefit. In this report, we demonstrate that aFGF, to a far greater degree than bFGF, enhances the growth of processes by cultured rat RGCs obtained from 7- to 12-day-old animals. Since by this age all of the centrally projecting processes of the RGCs have been fully formed (for references see ref. 18), at least some of this growth represents regeneration of neurites. The finding that bFGF did not result in a substantial increase in neurite outgrowth by RGCs suggests that aFGF and bFGF may work on separate populations of central neurons and that their effects might be mediated, at least in part, through distinct receptors.

MATERIALS AND METHODS

Fibroblast Growth Factors. aFGF and bFGF were purified from bovine retinas or brains by taking advantage of their affinity for heparin (10). Purification of aFGF to homogeneity was as described by Gospodarowicz *et al.* (19), yielding two biologically active forms (11, 21). bFGF was purified by the method of Lobb and Fett (20). Immunoblot analysis with monospecific antisera raised against synthetic peptides (22) was used to demonstrate that the aFGF and bFGF used in these studies were homogeneous and free from cross-contamination. One unit of activity of the fibroblast growth

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Abbreviations: aFGF and bFGF, acidic and basic fibroblast growth factors; GFAP, glial fibrillary acidic protein; RGC, retinal ganglion cell; CNS, central nervous system.

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factors is defined as the concentration required to obtain half-maximal incorporation of [^{3}H]thymidine by 3T3 cells (10) and is equivalent to ≈0.2 ng of the purified protein for aFGF and 0.05 ng for bFGF. To show that the preparations of growth factor used in these experiments were active, each batch of highly purified aFGF and bFGF was tested on 3T3 cells both before and after their use in RGC cultures.

Retinal Culture Methods. Retinas of Long Evans rat pups (age 7–12 days) were dissociated and cultured on glass coverslips as described (18). RGCs were identified with fluorescent dyes (18).

Quantification of RGC Survival and Regeneration of Processes. After 24 hr in culture, the RGCs were scored for survival and process outgrowth by the methods developed in this laboratory (18), with slight modifications. The survival of both solitary and clustered RGCs was assessed under phase-contrast optics by their bright appearance, the ability to accumulate fluorescein from fluorescein diacetate, and the presence of normal electrical activity (18, 23, 24). Retinal cultures were fixed with 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer. Process outgrowth by solitary RGCs was analyzed with phase-contrast microscopy by using a drawing tube to digitally encode the lengths with a bitpad and puck (Summagraphics) tied to a VAX 11/780 computer (Digital Equipment, Maynard, MA). Clustered RGCs could not be analyzed in this manner because many of their processes were hidden among the clumps of other retinal cells. It was necessary to quantify processes while visualizing them under the microscope and not from photographs because many small processes were often in a different focal plane from the cell body and could be easily overlooked if the focal length and ambient light were not continually varied.

RESULTS

Lack of Effect of aFGF and bFGF on RGC Survival After 24 hr in Culture. Compared with sibling postnatal cultures, the survival of RGCs after 1 day in culture did not appear to differ in medium alone or in medium containing bFGF or aFGF (even with the addition of heparin) (Fig. 1). This finding held true for both solitary and clustered RGCs. Survival at 24 hr was assessed since this time was chosen to quantify process outgrowth in previous studies (12).

aFGF Is Associated with Substantially More Process Outgrowth by RGCs than bFGF. After 1 day in culture, the solitary RGCs were scored for process regeneration. Fig. 2 presents photographs of representative RGCs grown in control medium and in medium containing 10 units/ml aFGF with heparin (10 μg/ml). On average a greater fraction of RGCs cultured in the presence of aFGF plus heparin grew neuronal processes, and the total length of processes per RGC was longer than in control medium. On the other hand, under these conditions treatment with bFGF did not substantially affect process outgrowth. Fig. 3*a* shows a dose–response curve for bFGF and for aFGF in the presence and absence of heparin (10 μg/ml). The control contained no added bFGF, aFGF, or heparin. In the absence of aFGF ≈30% of the solitary RGCs regenerated at least one process longer than the diameter of the cell body, similar to reported control findings (18). There was a graded increase in the proportion of RGCs with processes as aFGF was increased from 0.1 to 10 units/ml. At a larger dose (100 units/ml) this proportion began to decrease back toward the baseline, suggesting an inhibitory effect of higher concentrations of aFGF. Heparin enhanced the effects of aFGF at all concentrations by further increasing the percentage of ganglion cells that grew processes, but heparin alone had no effect. In addition, the total length of processes per solitary RGC

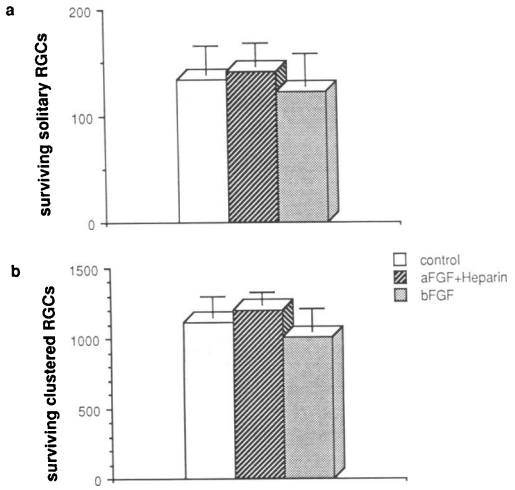

FIG. 1. Survival after 24 hr in culture of solitary (*a*) and clustered (*b*) RGCs grown on glass coverslips under control conditions, in the presence of aFGF (10–100 units/ml) plus heparin (10 μg/ml), or in bFGF (10–20 units/ml). Values are mean ± SEM of quadruplicate samples from three separate experiments. The number of RGCs represents the pooled data from the four coverslips for each experiment.

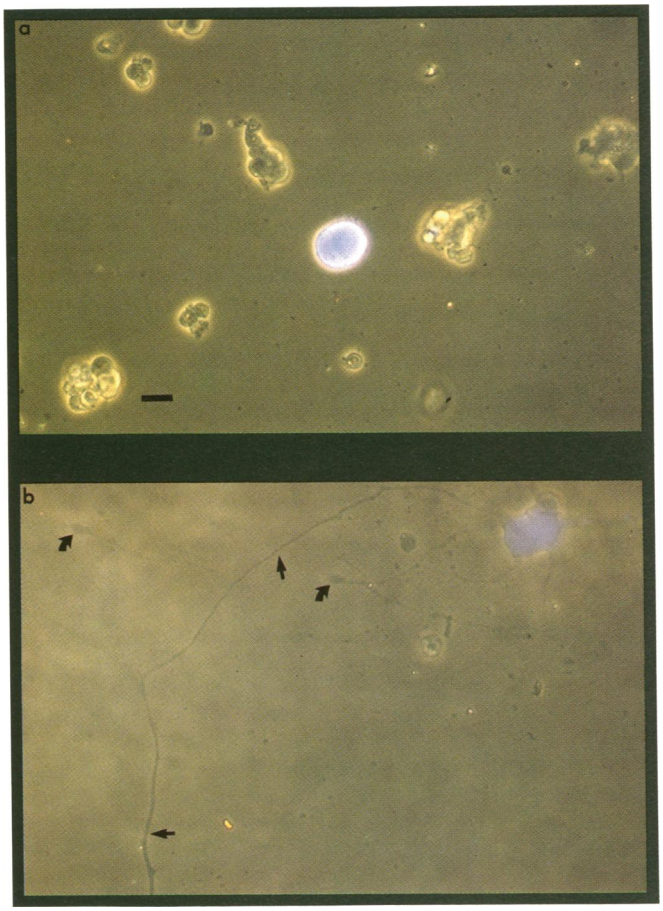

FIG. 2. Process outgrowth in 24-hr cultures of RGCs cultured on glass coverslips in aFGF (10 units/ml) plus heparin (10 μg/ml) (*b*) or in control conditions (*a*). The RGCs were labeled prior to dissociation by retrograde transport of the fluorescent dye granular blue (18). In *b* straight arrows mark the major cell process that extends out of the field, and curved arrows point to the growth cones of branches or smaller processes. Photographed with a combination of epifluorescence and phase-contrast microscopy. (Bar = 10 μm.)

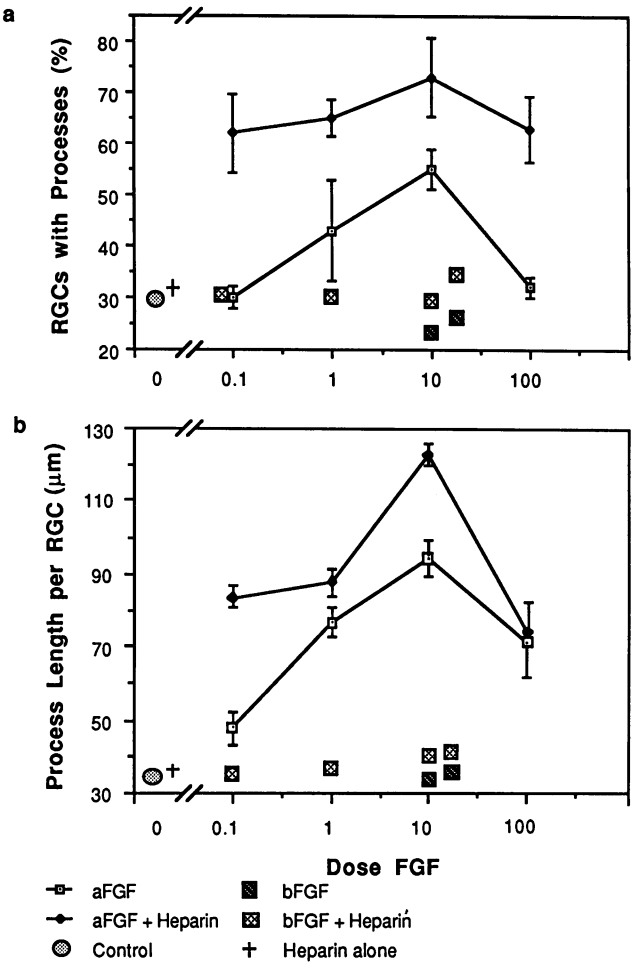

FIG. 3. Determination of aFGF concentration necessary for process outgrowth in the presence and absence of heparin (10 μg/ml). (*a*) Percentage of solitary RGCs that grew at least one process as long as the cell body is plotted versus the dose of aFGF (in units/ml) ± heparin, or bFGF (in units/ml) ± heparin. (*b*) Total length of processes per RGC is plotted versus the dose of aFGF (in units/ml) ± heparin, or bFGF (in units/ml) ± heparin. Data are shown for one of five experiments with similar results for aFGF. One of several experiments with bFGF is illustrated in which aFGF was also tested. Each experiment was based on quadruplicate platings of retinal cells. Values are means ± SEM; control cultures did not receive aFGF, bFGF, or heparin. The data for the percentage of RGCs growing processes (as in *a*) were analyzed with an extension of Fisher's exact test as described in ref. 18; the values for all concentrations illustrated of aFGF plus heparin were significantly greater than the control ($P < 0.0001$ after Bonferroni's correction). The results with heparin alone (10 μg/ml) or bFGF were not statistically different from the controls. Heparin (10 μg/ml) did not substantially enhance the effect of bFGF.

appeared to be significantly increased with increasing doses of aFGF up to 10 units/ml (Fig. 3*b*). At a dose of 100 units/ml, aFGF again appeared to be somewhat inhibitory compared with 10 units/ml. Heparin (10 μg/ml) potentiated the ability of aFGF to increase the length of processes per cell. When aFGF was inactivated by prolonged storage, it was no longer effective; the results were no different from the controls. In experiments in which aFGF stimulated neurite outgrowth, bFGF at 0.1, 1.0, 10, or 20 units/ml did not substantially enhance process outgrowth in sibling cultures in the presence or absence of heparin (Fig. 3).

aFGF Increases Neurite Initiation and Elongation. The fact that process outgrowth was enhanced by aFGF and heparin suggested that in the presence of aFGF the RGCs produced more processes, increased the elongation of existing processes, or did both. If the RGCs regenerated more processes in aFGF than did the control cells, then this finding would imply that the neurotrophic factor was in some way responsible for the initiation of growth of additional processes. If neurites were longer in the presence of aFGF than neurites in control cells, then aFGF might modulate the elongation of processes. In fact, the number of processes per RGC and the length of each process increased in cultures treated with aFGF plus heparin (Table 1 and Fig. 4).

Table 1 shows that RGCs cultured in aFGF plus heparin regenerated more processes per cell than RGCs in control medium. Since nothing is known of the form of the population of the number of processes per cell, it cannot be assumed to be normally distributed; thus, the data were analyzed with a nonparametric test. Even though such tests are less powerful than parametric statistics, the results were highly significant and showed that, in the presence of aFGF and heparin, RGCs produced more processes per cell than controls.

To use normal statistics to compare the length of each process in control medium and aFGF-containing medium, the lengths of processes would have to be normally distributed. Unfortunately, this was not the case; Fig. 4 *a* and *b* shows that a graph of the number of neuronal processes versus their individual lengths reveals a population skewed toward shorter process lengths. Nevertheless, a simple mathematical transformation of the data (taking the natural logarithm of the process lengths) resulted in a normal distribution (Fig. 4 *c* and *d* and S.A.L., unpublished observations). This manipulation permitted comparison by using the Student's *t* test of the mean natural logarithm (ln) of the length of each process growing from RGCs in control and in aFGF-treated medium. Fig. 4 *c* and *d* shows that significantly longer processes grew in medium containing aFGF plus heparin compared to control. Thus, aFGF and heparin produced elongation as well as initiation of neurites by RGCs.

Effect of aFGF plus Heparin on the Number of Glial Cells. Under the present conditions, glial cells, represented by Müller cells as well as by astrocytes, might be expected to be glial fibrillary acid protein (GFAP)-positive (25–28). When retinal cultures were incubated for 24 hr with aFGF (10 units/ml) and heparin (10 μg/ml), there was only a slight increase in the mean number of GFAP-positive cells. Control cultures contained 58 ± 9 glial cells, whereas aFGF plus

Table 1. Number of processes per RGC after 24 hr in control medium or in medium containing aFGF plus heparin

Exp.	aFGF, units/ml	Processes, no. per solitary RGC	
		Control	aFGF plus heparin
1	16	0.3	3.3
2	10	1.7	5.0
3	10	3.0	4.2
4	100	7.3	10.5
5	10	1.0	4.1
6	100	3.0	4.3
7	80	1.7	2.5
8	8	0.9	5.0
9	1	0.7	2.4
10	0.1	1.7	4.4
11	1	2.7	4.1
12	0.1	3.0	5.0

For each experiment 1 day after plating the retinal cultures, the number of processes per solitary RGC was counted in a double-blind manner for quadruplicate dishes in control medium and in medium containing aFGF (0.1–100 units/ml) plus heparin (10 μg/ml). In each experiment ≈160 solitary RGCs were observed, and all processes detectable at a magnification of ×500 under phase-contrast optics were scored. The number of processes per RGC in medium containing aFGF plus heparin was significantly greater than in the controls by the Sign test ($P < 0.001$).

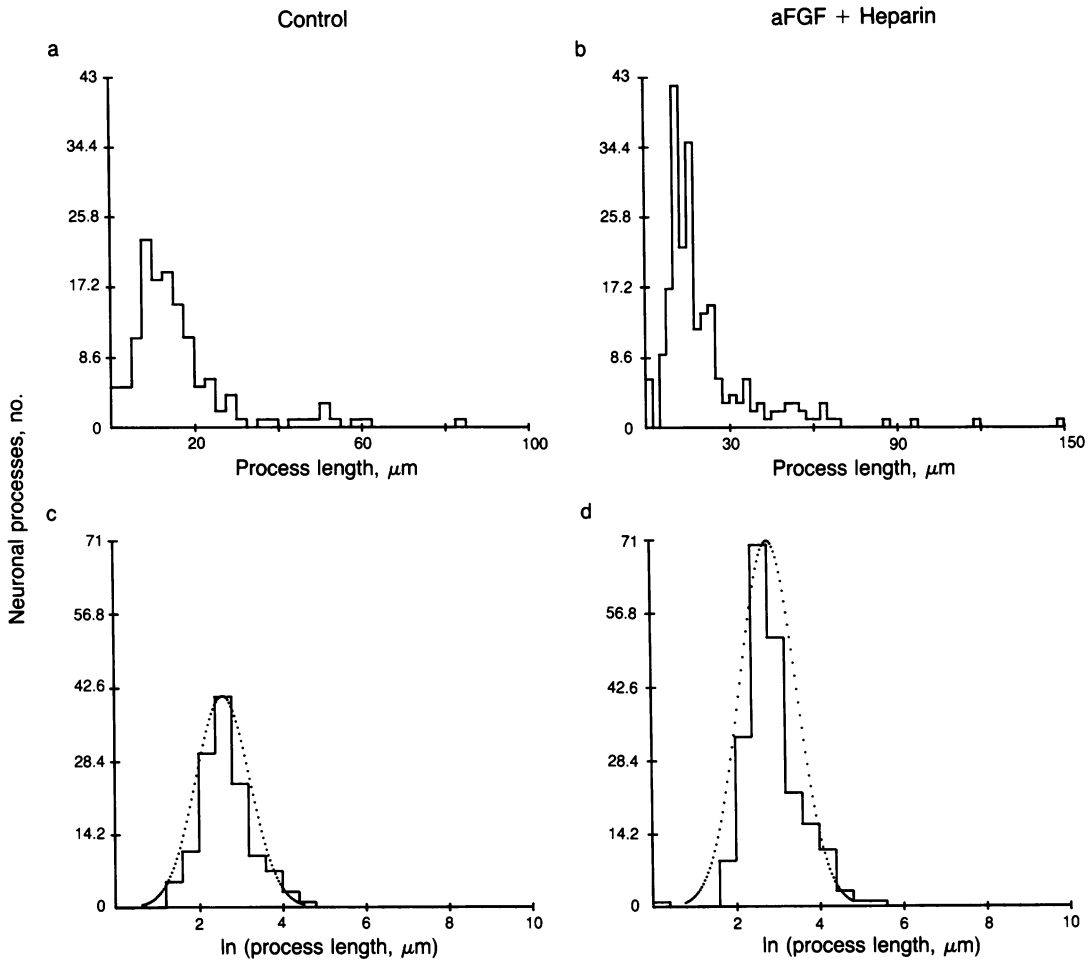

FIG. 4. Effect of aFGF (10 units/ml) plus heparin (10 μg/ml) on process length. The histograms represent the length of every neuronal process on 40 consecutive RGCs scored for outgrowth under control conditions (*a* and *c*) and in the presence of aFGF plus heparin in a representative experiment (*b* and *d*). For the controls, 137 processes were encountered on RGCs and analyzed; 223 processes were found in the aFGF plus heparin-treated cultures. (*a* and *b*) Frequency of occurrence is plotted as a function of the linear length of the neurites. In *a* the mean process length is 16.5 μm, and in *b* 22.7 μm. (*c* and *d*) Natural logarithm (ln) of the process lengths is displayed for the data in *a* and *b*, respectively. In *c* the mean ln value is 2.58, and the SD is 0.48, and in *d* the mean ln value is 2.80, and the SD is 0.50. The dotted lines represent the Gaussian fit to the data by the computer using the listed parameters. The mean ln value of the length of each process was significantly longer on RGCs plated in aFGF plus heparin compared to control ($P < 0.0005$ by the Student's *t* test). Four additional experiments yielded similar results.

heparin-treated cultures contained 68 ± 18 glial cells (mean ± SEM, $n = 10$ cultures in each case). This difference is not significant. Interestingly, the GFAP-positive cells in our retinal cultures were nearly always in intimate contact with clusters of retinal cells and only rarely with solitary RGCs. The observations were consistent with the hypothesis that the clustered retinal cells associated on the surface of the glial cells.

DISCUSSION

Several purified polypeptide growth factors have been reported to influence the growth of processes and survival of neurons in the mammalian CNS. These substances presumably interact with specific receptors on the cell surface that in turn trigger a series of intracellular events that may involve second messengers, ionic currents, and gene regulation (4). These growth factors include nerve growth factor (1, 29), neuroleukin (2), brain-derived growth factor (3), and bFGF (12, 14). Our findings constitute evidence that another peptide, a highly purified preparation of aFGF, has a profound influence on the elaboration of processes by a CNS neuron, the rat RGC. Interestingly, under the stated conditions various concentrations of bFGF did not substantially affect this system. In the present experiments 24 hr was selected as the criterion period to compare our results on process outgrowth induced by aFGF with studies involving bFGF (ref. 12 and see below). Although aFGF did not appear to influence the survival of RGCs after 24 hr *in vitro*, it is of course possible that the enhanced process growth represents the first manifestation of an increase in survival that will only become apparent when the retinal cultures are maintained for longer periods of time.

Our finding of an increase in neurite outgrowth by an identified, mammalian central neuron is noteworthy for three reasons. (*i*) By using fluorescent labels to identify the cells, a specific class of central neurons has been studied. (*ii*) Postnatal RGCs have been used, and thus process outgrowth in these neurons represents, at least in part, regeneration of axons in the mammalian CNS (18). (*iii*) Analysis of the number and the length of processes growing *in vitro* (Table 1 and Fig. 4) reveals that the addition of aFGF plus heparin resulted in an increase in both the initiation and elongation of neurites.

In the presence of heparin (10 μg/ml), half-maximal effects of aFGF on process outgrowth were observed at concentrations of <0.1 unit/ml (or 20 pg/ml), corresponding to ≈1 pM. This finding indicates a potency of aFGF for process outgrowth that may well exceed that of other polypeptide

growth factors such as nerve growth factor or bFGF. For example, Walicke *et al.* (12) reported that ≈1 pM bFGF was necessary for the half-maximal effect of prolonged survival of fetal rat hippocampal neurons *in vitro* but that 20 pM was required for the half-maximal influence on neurite extension at 24 hr. Also of importance, doses of bFGF (25–50 pM) that affected neurite outgrowth in embryonic cortical and hippocampal neurons (12, 14) had no substantial effect on RGC neurite outgrowth under our conditions.

The question arises whether or not the increase in process outgrowth by solitary RGCs observed in the present study could be influenced by the glia (GFAP-positive cells) in the cultures, expecially because aFGF leads to an increase in the number of astrocytes in other culture systems (9). It seems unlikely, however, that glial cells played a substantial role in the growth of processes by solitary RGCs for two reasons. (*i*) Comparing aFGF-treated cultures to control cultures, the number of glial cells was not significantly greater at 24 hr, the time chosen to study neurite outgrowth. Perhaps the apparent difference between this finding and that of previous studies (9) is due to the GFAP-positivity of Müller cells as well as of astrocytes under the present conditions. (*ii*) In general, solitary (in contradistinction to clustered) RGCs were not in contact with GFAP-positive cells. Thus, any effect of GFAP-positive cells on process regeneration by solitary RGCs would have to be mediated by a soluble factor that would have to be extremely potent to be able to act through the relatively large volume of the culture medium (2 ml). Since aFGF is a neurite extension factor for PC12 cells, a cloned line of neuroectodermal origin (11), there is a clear precedent for the action of aFGF directly on a neural cell. Nevertheless, it remains possible that aFGF acts on glial cells that in turn produce another factor that enhances process outgrowth by RGCs. Future studies that use the direct application of aFGF from a micropipette immediately adjacent to a solitary RGC while observing process growth (as described for nerve growth factor in ref. 30) may resolve the issue of direct aFGF effects on neurons.

The mechanism by which heparin potentiates aFGF is not known, but the possibility that aFGF interacts with a heparin-like molecule *in vivo* warrants discussion. Matthew and Patterson (31) have demonstrated that the monoclonal antibody INO binds to a laminin–heparan sulfate proteoglycan binding site and inhibits neurite outgrowth from sympathetic neurons. Interestingly, INO binds to the intact inner retina of adult rodents in the same distribution as the binding of Thy-1 monoclonal antibodies (V. Sehgal, L. Levin, and S.A.L., unpublished observations). Since Thy-1 antibodies had been shown to bind to the inner retina and RGCs (18, 32, 33), this finding is consistent with the notion that heparan sulfate proteoglycan in the extracellular matrix is in close apposition to RGCs. It is also known that aFGF has a high-affinity binding site for heparin-like molecules (10). Taken together, these results indicate that aFGF may bind in the mammalian retina to extracellular matrix molecules located in a position appropriate to influence process growth by RGCs *in vivo*. In the future it should be possible to test this hypothesis by determining if the regeneration of processes by RGCs can be influenced by aFGF in the living animal.

Interestingly, aFGF to a far greater degree than bFGF promotes the outgrowth of processes by postnatal RGCs while just the opposite appears to be true for fetal hippocampal neurons in culture (12, 15). These findings imply that these homologous growth factors have differential effects on disparate cell types in the mammalian CNS and indicate a previously unrecognized degree of specificity to their actions. Remaining issues to be settled include (*i*) whether or not aFGF and bFGF react with the same receptor (for a review see ref. 17), (*ii*) the exact distribution of these receptor(s) among different neuronal populations in the CNS, and (*iii*) the physiological role of these factors.

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